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Focus article

A preliminary study on the influence of cooking on the C and N isotopic composition of multiple organic fractions of fish (mackerel and haddock)

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ABSTRACT

Stable isotope analysis represents the principal scientific technique used in the reconstruction of ancient human diet. Characterisation of human diet requires that the isotopic baseline is established, i.e. the isotopic signals of consumed food groups. However, cooking may alter the bulk isotopic signal of food groups through the selective loss of macronutrients or biochemical components with different isotopic signals. In this study, we investigate the influence of cooking on the stable isotope values of raw flesh of two fish species (mackerel, with a high fat content, and haddock, having a low fat content) using three potential prehistoric cooking methods. The fish were boiled in a pot, grilled beside an open fire, and steamed in hot sand. Cooking times and temperatures were monitored. Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were measured on multiple fractions (bulk flesh, lipids, lipid-extracted flesh, water-extracted flesh, water-soluble compounds, and fish-bone collagen) before and after cooking. The results show that, for some fractions, cooking modified the composition, but changes in isotopic values relative to raw fish were in general <1‰. The results also show that isotopic signals of fish-bone collagen were not significantly altered during cooking, and confirm previous findings that showed significant isotopic offsets between fish-bone collagen and edible fish fractions.

1. Introduction

The main scientific tool used for diet reconstruction within archaeological research is the measurement of isotopic signals in different human bone fractions or biochemical components (Corr et al., 2005; Katzenberg, 2007; Lee-Thorp, 2008; Styring et al., 2010; Schwarcz and Schoeninger, 2012). The underlying principle is that different food groups may have specific isotopic signatures and that their consumption results in a signal transfer to the consumer, summarised in the well-known sentence "you are what you eat (plus a few permil)" (DeNiro and Epstein, 1976). In an archaeological context, the most common human isotopic measurements are performed on bone collagen nitrogen ($\delta^{15}N_{collagen}$) and carbon ($\delta^{13}C_{collagen}$). Nitrogen occurs primarily in food protein and the $\delta^{15}N_{collagen}$ signal is useful in establishing main protein sources (Hedges and Reynard, 2007). Bone collagen carbon is routed mainly from dietary protein, but it also includes a partial contribution from energetic macronutrients (lipids and carbohydrates), whereas bone bioapatite $\delta^{13}C$ ($\delta^{13}C_{bioapatite}$) reflects the carbon signal of the combined dietary mix (Hare et al., 1991; Ambrose and Norr, 1993; Tieszen and Fagre, 1993; Howland et al., 2003; Jim et al., 2004; Warinner and Tuross, 2009; Fernandes et al., 2012). Previous studies include the assessment of aquatic vs. terrestrial protein intake using both $\delta^{13}C_{collagen}$ and $\delta^{15}N_{collagen}$ (Schoeninger et al., 1983).

In archaeology, it is rare to find edible food remains whose isotopic signals can be measured directly. For terrestrial animal or fish foods, typically only bones, antlers and teeth are preserved (Szpak, 2011). However, isotopic signals of edible organs and tissues

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can be estimated from known isotopic offsets towards, for instance, bone collagen (Tieszen et al., 1983; Ramsay and Hobson, 1991; Hilderbrand et al., 1996; Piasentier et al., 2003). For fish, significant isotopic differences between flesh tissue and bone collagen have been reported ($\delta^{13}C_{flesh\text{-}bone}$ ca. −1 to −3‰, $\delta^{15}N_{flesh-bone}$ ca. +1 to +2‰)(Fischer et al., 2007; Sholto-Douglas et al., 1991). These offsets will vary and depend on the tissues and organs being compared, and result from differences in tissue-building biochemical pathways, and macronutrient and amino acid composition (Pinnegar and Polunin, 1999; Post et al., 2007; Logan et al., 2008). Differences in macronutrient isotopic composition in fish fat and protein are particularly significant, both within a single organism and among different fish species. Comparison of $\delta^{13}C$ values has shown that fish fat is typically more negative by 6–8‰ than fish protein (Post et al., 2007; Hoffman and Sutton, 2010).

Fish muscle homogenate is a heterogeneous system of proteins with fat particles dispersed in an aqueous phase (Thawornchinsombut and Park, 2006). Fish muscle proteins are classified according to their solubility (Haard, 1992). Sarcoplasmic proteins (myogens), accounting for 10–25% of muscle proteins, are soluble in an aqueous solution with an ionic strength below 0.15. Myofibrillar proteins, mostly myosin and actin, accounting for 70–90% of muscle proteins, are soluble in an aqueous solution with an ionic strength above 0.15. Stomal proteins (e.g. collagen), accounting for 3–10% of muscle proteins, form the connective tissue in fish muscle and are water-insoluble. Non-protein nitrogen (NPN) compounds (Waarde, 1988), which are extractable in an aqueous solution, are also present in fish muscle. For teleosts (Fraser et al., 1961) such as haddock and mackerel, NPN compounds typically account for up to 20% of total fish muscle nitrogen (Velankar and Govindan, 1958). In mackerel, about 25% of non-protein compounds are free amino acids; the remaining NPN compounds include peptides, nucleotides, creatine, betaines, trimethylamine oxide, urea, and ammonia (Fine, 1992). A similar distribution in NPN compounds is observed for haddock (Shewan et al., 1952). Fish cooking usually leads to a loss of muscle moisture and the following fraction loss sequence may be hypothesized: NPN compounds, sarcoplasmic proteins, myofibrillar proteins, and stomal proteins. High temperatures will cause protein denaturation; proteins may then aggregate, resulting in a decrease in protein solubility (Camire, 1991). Protein aggregation will probably further increase the selective loss of NPN compounds, but published cooking experiments usually only report changes in crude protein content, determined from total nitrogen values (Salo-Väänänen and Koivistoinen, 1996). Selective loss of specific amino acids as an additional source of isotopic changes seems unlikely. Although there are large differences in isotopic values among different amino acids in aquatic animals (Styring et al., 2010), significant alterations in amino acid profiles between raw and cooked fish specimens have not been reported (Castrillón et al., 1996; Oluwaniyi et al., 2010).

The nutritional properties of raw fish are well-known to undergo changes during cooking (Bognár, 1998; García-Arias et al., 2003; Gokoglu et al., 2004; Baylan et al., 2011; Oduro et al., 2011; Oluwaniyi and Dosumu, 2009; Oluwaniyi et al., 2010). These changes include modifications in the protein to lipid ratio. In most cooking methods, except for frying, there is an increase in protein over lipid content, but the overall pattern is complex, varying with cooking method and fish species. Given the differences in $\delta^{13}C$ values between fish protein and lipids, it is expected that cooking will also result in modifications of bulk flesh isotopic signals. Furthermore, differences in isotopic signals among fish fractions (e.g. protein types, single compounds) and selective losses during cooking could result in changes in macronutrient isotopic values. Such isotopic shifts with some degree of variability are observed, for instance, in different fish lipid extraction protocols both for $\delta^{13}C$ and $\delta^{15}N$ signals (Logan et al., 2008; Hoffman and Sutton, 2010; Miller et al., 2010).

The use of isotopic data from archaeological fish bones for human paleodietary reconstruction presents three different basic problems. The first is whether the isotopic signal of the archaeological fish bone corresponds to its original signal. Any isotopic shift may reflect the effects of cooking methods and diagenesis, aspects addressed elsewhere (Stewart and Gifford-Gonzalez, 1994; Nicholson, 1996). The second problem is the relationship between a fish-bone isotopic signal (e.g. $\delta^{13}C_{collagen}$) and the isotopic signal of edible raw fish portions (e.g. flesh bulk $\delta^{13}C$ or protein $\delta^{13}C$) (Sholto-Douglas et al., 1991; Fischer et al., 2007). The final question is how the isotopic values of edible portions of raw fish may be influenced by different cooking methods. Here, we are primarily interested in the last point, although some aspects of the first two points will also be addressed.

The main objective of the research presented here was to undertake a pilot study to illustrate potential isotopic shifts in fish flesh and isolated components as a result of cooking, following methods potentially used in the past. The working hypothesis was that any isotopic changes would be mainly associated with the macronutrient composition of the species selected for study. Thus, two fish specimens having a low and a high fat content were used in this study.

2. Materials and methods

2.1. Fish specimens

Two fish specimens were selected for study, a North Atlantic haddock (*Melanogrammus aeglefinus*) and an Atlantic mackerel (*cf. Scomber scombrus*), both with a length of ca. 35 cm. Haddock is classified as a lean fish, with a fat content below 2% of total weight, while Atlantic mackerel is classified as a high-fat fish, with a fat content above 8% of total weight (Ackman, 1994). Both specimens were bought in October 2011, a season when Atlantic mackerel has a near maximum fat content (ca. 20% fat by weight) (Wallace, 1991). Fish specimens were cooked the day after purchase.

From both specimens, portions of flesh of similar weight (ca. 70 g) were cut and cooked using three different methods. After cooking, aliquots of white and dark fish flesh were removed, freeze-dried and submitted to elemental and isotopic analysis.

2.2. Cooking methods

Portions of both fish specimens were cooked using three different methods, which may have been used prehistorically without leaving unambiguous archaeological evidence:

2.2.1. Steamed

This cooking method (Fig. 1) represented an exercise in experimental archaeology with a hypothetical reconstruction based on archaeological evidence from north-German Mesolithic sites (Lage, 2004). The setup consisted of a dried clay base over a fire, to stabilize temperature, with fish cooked in hot sand on top of the clay base (Fig. 1a). Two fish portions, one for each species, were first wrapped in tree leaves and then in clay (Fig. 1b). Clay parcels were placed in hot sand (Fig. 1c). The temperature of the clay base and sand was monitored using a thermocouple. Temperatures were extremely variable both for the clay base (300–400 °C), and sand (90–170 °C). However, temperature measured at the fish surface indicated a constant temperature of 100 °C, with the fish effectively steaming in its own moisture and that of the leaves and clay. The fish was cooked for approximately 30 min and removed from the sand when the cooking temperature started rising (Fig. 1d). The

Fig. 1. Experimental setup for cooking fish, derived from Mesolithic archaeological finds from northern Germany (Lage, 2004). The setup consisted of a dried clay disk over which sand was deposited (a). Fish samples were wrapped in leaves and then in clay (b). Fish was steamed for approximately 30 min (c). Samples were removed (d), and the fish tasted for palatability (e).

clay wrapping was broken and the fish tested for palatability (Fig. 1e).

2.2.2. Grilled

From each fish specimen a portion was directly exposed to radiant heat of a fire at a distance of ca. 25 cm. Temperature monitoring using a thermocouple at the fish surface showed temperatures having a wide range, from 100 to 200 °C, varying according to wind direction and fire intensity. The fish was cooked for approximately 30 min.

2.2.3. Boiled

From each fish specimen a portion was placed in a pot with boiling water and left cooking for 25 min. There is now abundant evidence that fish was cooked in prehistoric ceramic pots (Craig et al., 2011), presumably by boiling on an open fire, and it is generally assumed that aceramic societies could have boiled food in organic containers by dropping heated rocks in the cooking water.

2.3. Isotopic and elemental analysis

Stable isotope analyses of nitrogen and carbon were performed at the stable isotope laboratory of the Museum für Naturkunde, Berlin, with a THERMO/Finnigan MAT V isotope ratio mass spectrometer, coupled to a THERMO Flash EA 1112 elemental analyser via a THERMO/Finnigan Conflo III- interface.

For each element (X) stable isotope values ($\delta^{n}X$) were expressed using standard notation ($\delta^{13}C$ and $\delta^{15}N$) in parts per mil (‰), defined as $\delta^{n}X = ((R_{sample}/R_{standard}) - 1) \times 1000$ (‰), where R_{sample} is the isotopic ratio measured in the sample and $R_{standard}$ is the same isotopic ratio in the standard. For $\delta^{15}N$, the standard was atmospheric nitrogen (Mariotti, 1983), and for $\delta^{13}C$ the standard was VPDB (Vienna PeeDee Belemnite standard).

Instrumental uncertainty, based on repeated measurements of laboratory standard material (peptone), was in general better than 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$. Duplicate measurements on separate aliquots of bulk mackerel and haddock flesh, cooked and uncooked, scatter however significantly more than expected from this instrumental uncertainty. The standard deviation calculated from seven measurement pairs is ca. 0.5‰. This larger uncertainty probably reflects the difficulty of obtaining a representative milligram-sized sample of inhomogeneous material, and is taken into account in the discussion.

2.4. Analysed fish fractions

2.4.1. Bulk fish flesh

Three aliquots of raw and cooked bulk white muscle were collected from different areas of each fish portion. For both haddock and mackerel, white muscle samples were observed under an optical microscope to ensure that they did not contain any dark muscle. For mackerel, samples of dark muscle were also collected. Flesh samples were dried under a continuous air flow at room temperature.

2.4.2. Lipid-extracted flesh and lipids

Previously dried fish aliquots were minced into a pulp in a solvent mixture (20 ml per gram of sample) of dichloromethane/methanol (2:1). The samples were ultrasonicated (2 × 30 min) and then centrifuged. The solvent mix was transferred to a separate container by a combination of siphoning and decanting. Lipid-extracted flesh samples were dried under a continuous air flow. Lipids were recovered by evaporating the siphoned-off solution under a nitrogen flow.

2.4.3. Water-extracted flesh and water-soluble compounds

Dried aliquots of bulk fish flesh were minced into a pulp in Milli-Q water and left in solution for 24 h at room temperature. Water-soluble compounds were isolated by decanting the water through a 1 μm quartz filter to limit contamination by particles of fish flesh. Both fractions, water-extracted flesh and water-soluble compounds, were dried under a continuous air flow at room temperature.

2.4.4. Fish-bone collagen

Fish bones were mechanically cleaned of adhering flesh and then placed for 1 h in a 12% solution of sodium hypochlorite (NaClO) to oxidize any remaining surface organics. The samples were demineralised in a solution of 1 M HCl during 30 min, then rinsed several times under centrifugation to neutrality using Milli-Q water. The collagen pellet was resuspended in a solution of HCl (pH = 3) and gelatinised in a hot water bath (85 °C) overnight. The

gelatine solution was frozen using liquid nitrogen and then freeze-dried.

3. Results & discussion

Isotopic results and C/N values for different fractions and cooking methods are listed in Table 1. Figs. 2 and 3 show the results for carbon and nitrogen isotopes respectively. As can be seen, the effect of cooking on the $\delta^{13}C$ and $\delta^{15}N$ values of the different fractions is generally small and usually not larger than the variability observed in single tissue measurements, associated with compositional inhomogeneities (Pinnegar and Polunin, 1999).

The differences between cooked and uncooked mackerel bulk flesh $\delta^{13}C$ values were smaller than 0.5‰; for haddock these were only 0.1‰ (Fig. 2). The other fractions showed maximum $\delta^{13}C$ differences between cooked and raw mackerel values of 0.7‰ for bone collagen, 0.6‰ for lipid-extracted flesh, and 0.9‰ for water-extracted flesh. For haddock these differences were smaller: 0.2‰ for bone collagen, 0.2‰ for lipid-extracted flesh, and 0.8‰ for water-extracted flesh. The larger variability observed for mackerel may be attributable to its high content of isotopically light lipids ("dark-muscle raw" $\delta^{13}C$: −28.2‰; extracted lipids: −27.5‰), also reflected in the higher C/N values for bulk and water-extracted flesh (Table 1). Considering the measurement scatter of up to 0.5‰ within samples, none of these differences are statistically significant at the 2-sigma (95%) level. For both mackerel and haddock, the differences in $\delta^{15}N$ values between raw and cooked fractions are smaller than 1.2‰ and not statistically significant.

Potential modifications in isotopic values during cooking are due to the preferential loss of compounds having different physical and chemical properties and isotopic signals. The results presented here confirm the large difference in $\delta^{13}C$ values (ca. 8‰) between fish lipids and lipid-extracted raw flesh observed in previous studies (Post et al., 2007; Hoffman and Sutton, 2010). Significant isotopic differences were also observed between other fractions, such as water-soluble compounds and lipid-extracted flesh. Comparison of the $\delta^{13}C$ values for the different fractions of the lean haddock and fatty mackerel shows that haddock fractions are heavier. However, when the lipids are separated from the lipid-extracted fraction, the $\delta^{13}C$ spacing between haddock and mackerel for lipid-extracted flesh, lipids, and bone collagen are similar (2.2‰, 2.4‰, and 2.2‰, respectively). The larger differences observed for the bulk and water-extracted flesh (4.8‰ and 4.1‰) reflect the larger proportion of lipids in the mackerel. The apparent correlation between more negative $\delta^{13}C$ and higher C/N values is also consistent with relative enrichment in carbon-rich lipids. Considering the $\delta^{13}C$ difference between bulk and lipid-extracted flesh, a simple mass balance suggests a 21% lipid content in the mackerel bulk flesh, close to the ca 20% maximum expected for October (Wallace, 1991). Although this comparison, fortuitously, produces excellent agreement, the uncertainty in the results of this pilot study means that true mass balance calculations are not warranted. The qualitative consistency of the changes observed nevertheless supports the reliability of the results presented.

The implications of these results in consumer diet reconstruction studies depend on the specific dietary proxy (e.g. $\delta^{13}C_{collagen}$, $\delta^{15}N_{collagen}$, $\delta^{13}C_{bioapatite}$) and on which food fractions (e.g. bulk, protein, lipids) determine the isotopic proxy signal. Bulk carbon isotopic values of food groups (e.g. whole flesh) determine the bone $\delta^{13}C_{bioapatite}$ signal in mammalian consumers (Fernandes et al., 2012). The results show that in spite of the significant isotopic differences between fish fractions, isotopic changes in bulk flesh during cooking were smaller than ca. 1‰. This implies that preferential losses of certain fractions or compounds were not sufficiently large to meaningfully modify isotopic values. Thus, there is no clearly observable pattern linking preferential loss of certain compounds and cooking-induced isotopic variations. At the macronutrient level, lipid and protein isotopic signals (lipid-extracted flesh) also did not undergo isotopic changes larger than ca. 1‰ when compared with raw fish macronutrients. This is of significance in diet reconstruction studies of mammalian consumers relying on $\delta^{13}C_{collagen}$ and $\delta^{15}N_{collagen}$, as protein is the principal macronutrient determining these signals.

Nevertheless, the results presented here have some implications for ancient diet reconstruction studies. Fish bones are often retrieved from the archaeological record and extracted collagen may be used to define an isotopic baseline in human dietary studies. Our results show that cooking does not introduce significant isotopic modifications in fish-bone collagen, in agreement with previous results on mammalian bones (DeNiro et al., 1985). Nevertheless, for fish cooked using the methods described here, an uncertainty of up to 1‰ needs to be considered when establishing the isotopic baseline of fish in human diet reconstruction studies. A similar observation applies to ecological studies that rely on fish bones recovered from refuse deposits (Misarti et al., 2009; Szpak et al., 2013). Furthermore, ancient diet reconstruction studies need to consider the isotopic offsets between the fractions recovered from the archaeological record (e.g. collagen) and relevant edible fractions (e.g. protein, bulk flesh). The results show that fish-bone collagen $\delta^{13}C$ is ca. 10‰ heavier than lipids and ca. 2‰ heavier than the lipid-extracted flesh for both haddock and mackerel. However, it is ca. 3.5‰ heavier than bulk flesh in mackerel and ca. 2.5‰ in the haddock as a result of their different fat contents. A comparison of the $\delta^{15}N$ of fish-bone collagen and bulk flesh shows

Table 1
Isotopic and atomic elemental ratios (C/N) measured in different extracted fractions for each fish specimen and cooking method. Duplicate measurements set the uncertainty at 0.5‰.

Fraction	Cooking method	Haddock			Mackerel		
		$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	C/N	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	C/N
Bulk flesh[a]	Raw	14.8	−17.9	3.4	10.3	−21.1	5.6
	Boiled	15.2	−18.0	3.2	10.6	−21.0	5.7
	Grilled	14.9	−18.0	3.5	10.1	−21.5	6.2
	Steamed	14.8	−18.0	3.5	10.7	−21.4	6.5
Fish-bone collagen	Raw	13.8	−15.5	2.9	10.2	−17.9	3.2
	Boiled	13.1	−15.3	3.0	10.5	−18.0	3.0
	Grilled	13.5	−15.3	3.0	10.0	−17.2	2.9
	Steamed	13.4	−15.3	3.0	10.1	−17.4	2.9
Lipid-extracted flesh	Raw	16.2	−17.1	3.3	9.8	−19.2	3.3
	Boiled	15.1	−17.3	3.4	10.1	−19.5	3.5
	Grilled	15.0	−17.3	3.4	10.5	−19.3	3.5
	Steamed	16.0	−17.1	3.4	10.0	−19.8	3.8
Lipid[b]	Raw	–	−24.5	–	–	−27.4	–
	Boiled	–	−25.5	–	–	−27.5	–
	Grilled	–	−25.3	–	–	−27.3	–
	Steamed	–	−25.1	–	–	−27.7	–
Water-extracted flesh	Raw	16.0	−17.7	3.6	11.0	−21.9	5.1
	Boiled	15.6	−18.2	3.6	–	−22.8	6.6
	Grilled	16.4	−18.0	3.8	10.2	−22.3	5.4
	Steamed	15.2	−17.4	3.6	10.5	−21.5	4.8
Water-soluble[b]	Raw	14.2	−17.7	3.5	6.5	–	–
	Boiled	10.6	−19.1	2.6	5.1	–	–
	Grilled	8.6	−19.1	2.7	4.0	–	–
	Steamed	11.1	−19.4	2.6	3.9	–	–
Dark-muscle	Raw	–	–	–	10.5	−28.2	16.7

[a] Isotopic values of haddock bulk flesh are based on one set of measurements. A second set was rejected because of internal inconsistencies.
[b] Extracted lipids contained insufficient nitrogen for $\delta^{15}N$ measurement; no reliable $\delta^{13}C$ or C/N values were obtained for mackerel water-soluble fraction.

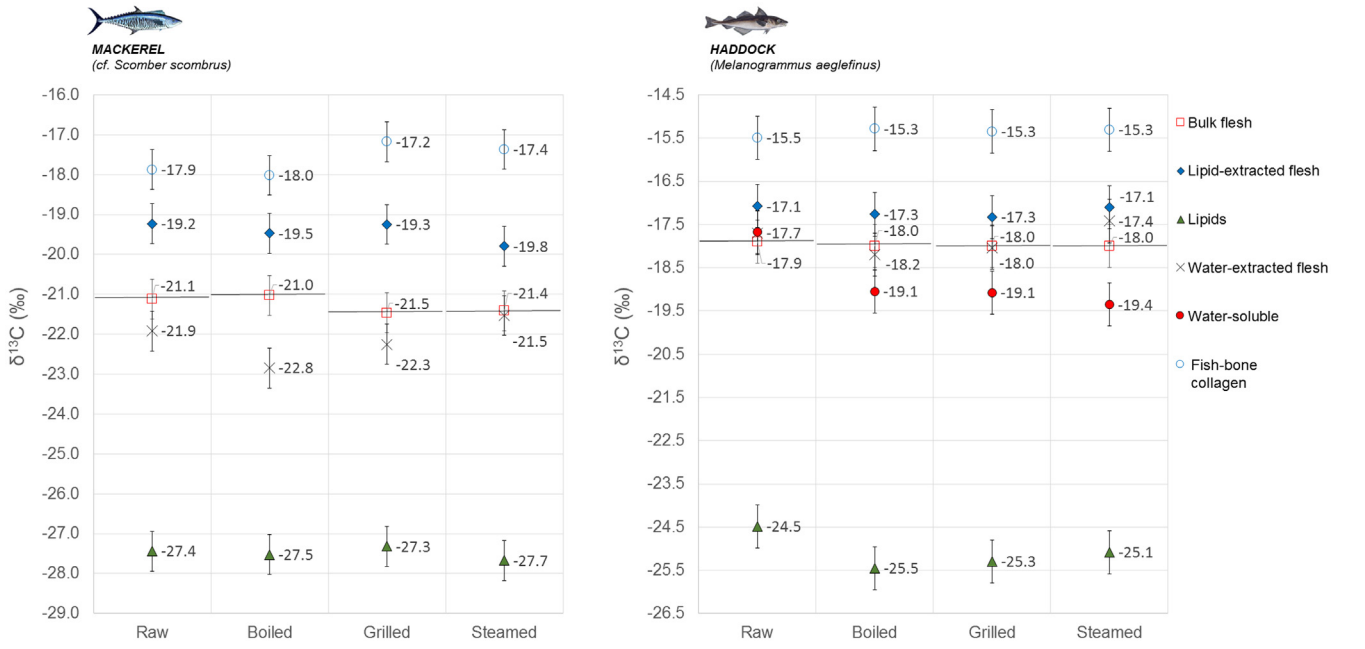

Fig. 2. Carbon isotopic values ($\delta^{13}C$) measured in different fractions (bulk flesh, lipid-extracted flesh, water-extracted flesh, fish-bone collagen, lipids, water-soluble organics) for each fish specimen and cooking method. Error bars correspond to 1-sigma uncertainty. The horizontal lines reference the bulk flesh value. Fish pictures from Wikimedia (users Robbie Cada and NOAA FishWatch).

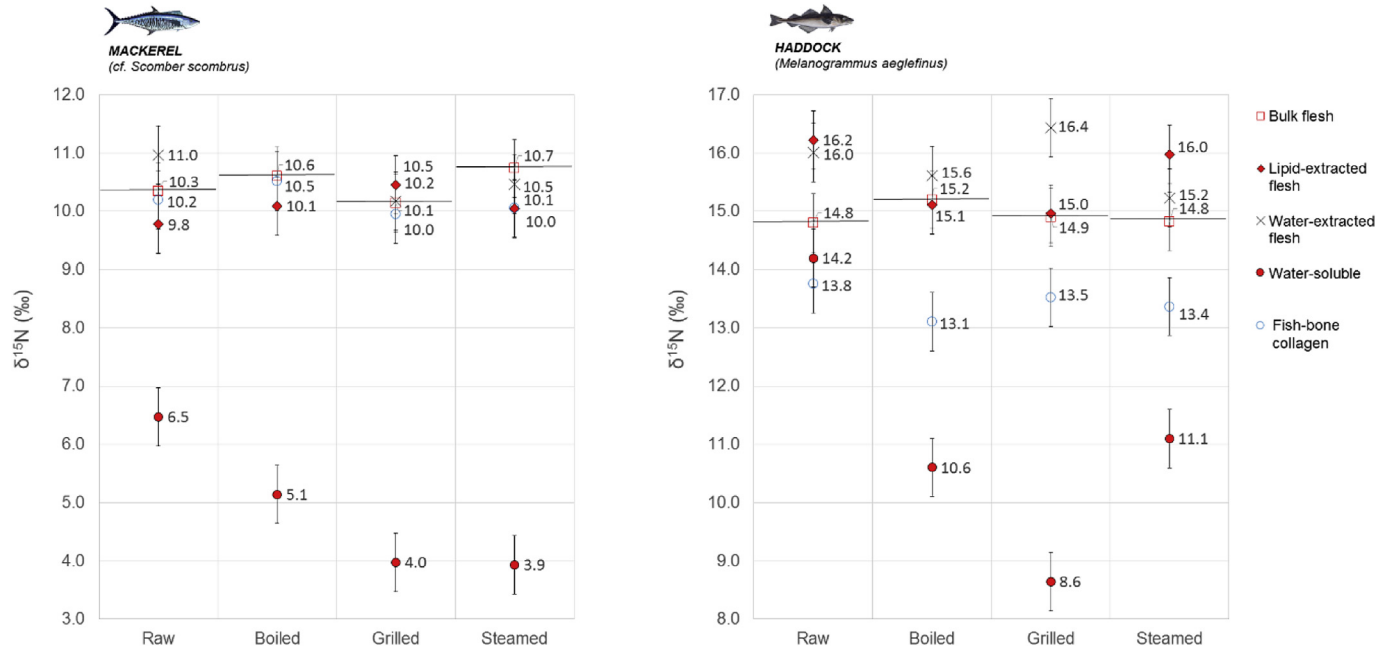

Fig. 3. Nitrogen isotopic values ($\delta^{15}N$) measured in different fractions (bulk flesh, lipid-extracted flesh, water-extracted flesh, water-soluble compounds, and fish-bone collagen) for each fish specimen and cooking method. Error bars correspond to 1-sigma uncertainty. The horizontal lines reference the bulk flesh value. Fish pictures from Wikimedia (users Robbie Cada and NOAA FishWatch).

that there is no significant difference for mackerel, while for haddock, bulk flesh $\delta^{15}N$ values are 1–2‰ higher than in fish-bone collagen.

The results of this pilot study emphasize the need for additional research, extended to include other fish species, new food groups (e.g. meat of terrestrial animals, plant foods), and different cooking methods. Cooking methods based on evidence from the archaeological and historical record will be of particular interest. Other research should aim at isolating different food macronutrients and compounds, characterising their isotopic signal, and the extent to which these are metabolized. This will offer a better understanding of the underlying causes associated with cooking-induced isotopic changes and improve our knowledge of isotopic offsets between edible food fractions and recovered archaeological material, such as the isotopic offsets between (e.g.) bone collagen and meat in mammals.

4. Conclusions

Modifications to $\delta^{13}C$ and $\delta^{15}N$ caused by different cooking methods were smaller than ca. 1‰ for defined fractions of mackerel and haddock, and were not statistically significant. In agreement with previous research, isotopic differences were observed between bone collagen and edible fish fractions. For both mackerel and haddock, $\delta^{13}C$ values were ca. 10‰ higher in fish-bone collagen than in lipids and ca. 2‰ higher than in lipid-extracted flesh. Differences in fat content resulted in varying $\delta^{13}C$ offsets between bulk flesh and fish-bone collagen. The $\delta^{15}N$ values of mackerel collagen and lipid-extracted flesh were similar, whereas haddock collagen $\delta^{15}N$ was ca. 2‰ lower than in lipid-extracted flesh. These offsets are relevant in ancient diet reconstruction studies given the significant differences between the isotopic signals of edible fractions and fish-bone collagen. Furthermore, the results indicate that an additional isotopic uncertainty of up ca. 1‰ associated with fish cooking needs to be considered when defining an isotopic baseline in diet reconstruction studies.

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